

Cytochrome b_5 Coexpression Increases *Tetrahymena thermophila* $\Delta 6$ Fatty Acid Desaturase Activity in *Saccharomyces cerevisiae*

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Very-long-chain polyunsaturated fatty acids such as arachidonic, eicosapentaenoic, and docosahexaenoic acids, are important to the physiology of many microorganisms and metazoans and are vital to human development and health. The production of these and related fatty acids depends on $\Delta 6$ desaturases, the final components of an electron transfer chain that introduces double bonds into 18-carbon fatty acid chains. When a $\Delta 6$ desaturase identified from the ciliated protist *Tetrahymena thermophila* was expressed in *Saccharomyces cerevisiae* cultures supplemented with the 18:2 $^{\Delta 9,12}$ substrate, only 4% of the incorporated substrate was desaturated. Cytochrome b_5 protein sequences identified from the genome of *T. thermophila* included one sequence with two conserved cytochrome b_5 domains. Desaturation by the $\Delta 6$ enzyme increased as much as 10-fold when *T. thermophila* cytochrome b_5 s were coexpressed with the desaturase. Coexpression of a cytochrome b_5 from *Arabidopsis thaliana* with the $\Delta 6$ enzyme also increased desaturation. A split ubiquitin growth assay indicated that the strength of interaction between cytochrome b_5 proteins and the desaturase plays a vital role in fatty acid desaturase activity, illustrating the importance of protein-protein interactions in this enzyme activity.

Fatty acid desaturases are enzymes that introduce double bonds into fatty acid hydrocarbon chains at specific locations; the actual substrate of the reactions are fatty acids esterified to a carrier molecule such as coenzyme A, acyl carrier protein, or the glycerol molecule of a lipid. Most desaturases additionally exhibit strong preference with regard to the length of the substrate hydrocarbon chain and to the location of double bonds already present in the chain. The $\Delta 6$ desaturases preferentially use 18-carbon substrates already desaturated at the $\Delta 9$ and $\Delta 12$ carbons, producing 18:3 $^{\Delta 6,9,12}$ as their product. These desaturases are members of a broad family of membrane-bound enzymes that acquire the electrons from an electron transport chain composed of NADH:cytochrome b_5 reductase and cytochrome b_5 (1, 2). The $\Delta 6$ enzymes are members of a subfamily of desaturases whose protein sequence includes a domain homologous to cytochrome b_5 ; all eukaryotic $\Delta 6$ desaturases contain this cytochrome b_5 -like domain. By itself, cytochrome b_5 is a small electron carrier protein composed of a hydrophobic domain that anchors the protein in the membrane, coupled to a hydrophilic domain, which noncovalently binds a heme group (3); the highly conserved heme-binding sequence motifs are the model for the electron transfer domains identified in $\Delta 6$ and other desaturases.

The $\Delta 6$ desaturases take part in synthesis of very long chain polyunsaturated fatty acids including 20:4 $^{\Delta 5,8,11,14}$ (arachidonic acid [ARA]), 20:5 $^{\Delta 5,8,11,14,17}$ (eicosapentaenoic acid [EPA]), and 22:6 $^{\Delta 4,7,10,13,16,19}$ (docosahexaenoic acid [DHA]) that have important biological roles in membrane structure and fluidity. These desaturases are found in a wide range of organisms; many are found in marine microorganisms rich in polyunsaturates, including cyanobacteria and phytoplankton (4). Besides the role that desaturases play in providing fatty acids necessary for membrane structure within these organisms, the polyunsaturated fatty acids produced also form the base of a food chain that supports marine invertebrates, fish, and marine mammals. These metazoan organisms, many of which are themselves capable of $\Delta 6$ desaturation, are valuable both for their contributions to marine environments

and because they contribute an important component of human diet.

Polyunsaturated 20- and 22-carbon fatty acids are important constituents of mammalian tissues, particularly of neural and reproductive tissue, and serve as precursors to the synthesis of signaling molecules such as leukotrienes and prostaglandins (5). The requirement for these fatty acids is met mainly by dietary intake at all stages of life; the requirements are particularly critical early in development (6). Declining fish populations and increasing toxin accumulation in fish make it desirable to develop new sources of polyunsaturated fatty acids (7). There is great potential for polyunsaturated fatty acid synthesis through expression of the necessary genes in yeast or plants, but most attempts at creating useful fatty acids have failed to yield high quantities of the desired products (8, 9). One cause of the low yield may be that heterologously expressed proteins do not interact efficiently with the host proteins, leading to low biochemical activity.

The ciliated protist *T. thermophila* has three known desaturase activities; $\Delta 9$ desaturation of 18:0 to 18:1 $^{\Delta 9}$, $\Delta 12$ desaturation of the 18:1 $^{\Delta 9}$ to 18:2 $^{\Delta 9,12}$, and $\Delta 6$ desaturation of 18:2 $^{\Delta 9,12}$ to 18:3 $^{\Delta 6,9,12}$ (10). The fatty acid composition of this organism has been investigated for many years because the level of desaturation in its membranes changes rapidly with changes in growth temperature (11, 12). In this work, proteins important to *T. thermophila* $\Delta 6$

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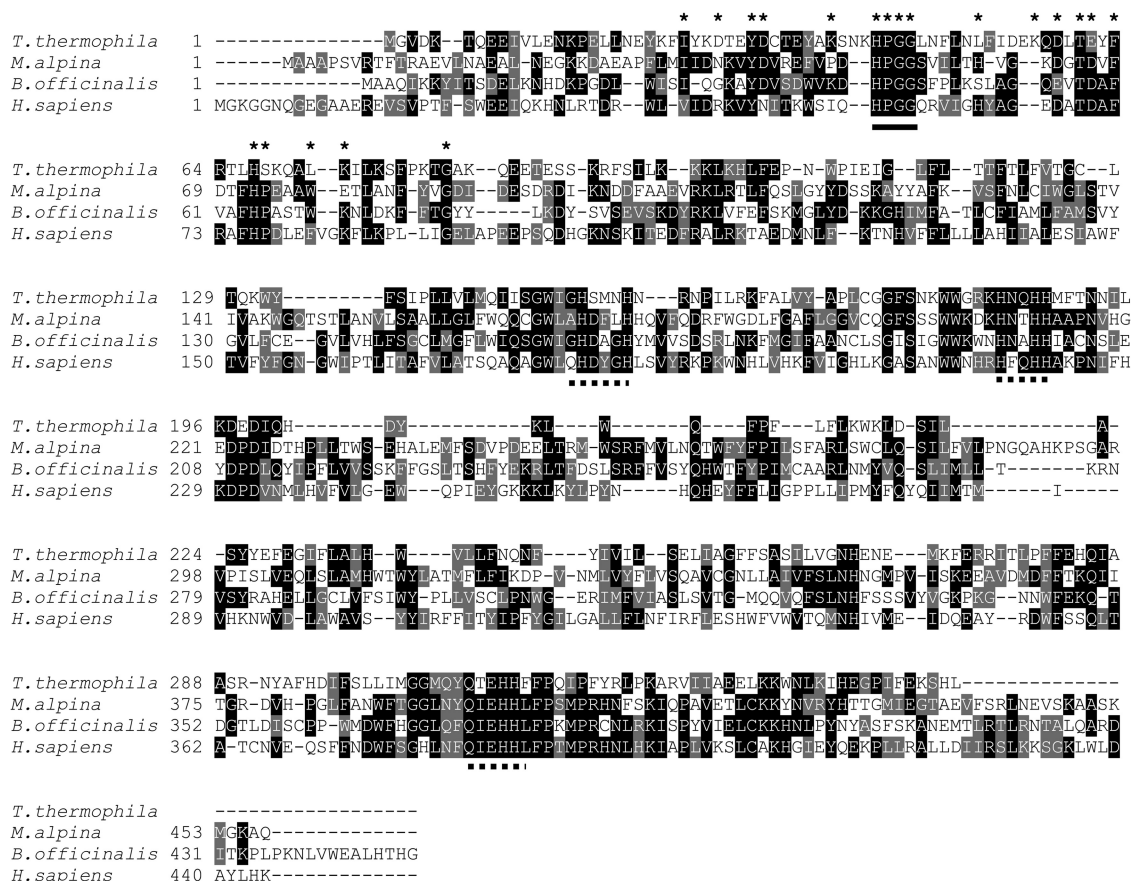


FIG 1 Amino acid sequence comparison of the *T. thermophila* Δ6 desaturase with other known Δ6 desaturases. The *T. thermophila* candidate Δ6 desaturase amino acid sequence (THERM_00138530) is compared to known Δ6 desaturase sequences from *Mortierella alpina* (AAF08685), *Borago officinalis* (AAC49700), and *Homo sapiens* (NP_004256). The three conserved histidine boxes have a dotted underline, and the conserved HPGG motif in the N-terminal cytochrome *b*₅ domain has a solid underline; the first histidine residue in that motif is the site of the H42A mutation (see the text for details). The asterisks (*) indicate residues of Des6 identical to the consensus cytochrome *b*₅ motif (25).

fatty acid desaturase activity have been identified and functionally characterized through heterologous expression in *S. cerevisiae*. When initial expression of the Δ6 desaturase from *T. thermophila* produced only low desaturase activity in the yeast heterologous system, identification of cytochrome *b*₅s from *T. thermophila* and their coexpression with the desaturase in yeast produced a marked increase in desaturation. Split ubiquitin growth assays demonstrated that the physical interactions between the cytochrome *b*₅ proteins and the *T. thermophila* desaturase play an important role in fatty acid desaturation. These findings emphasize the importance of understanding protein-protein interactions required to optimize enzyme activity.

MATERIALS AND METHODS

Cloning of a Δ6 desaturase. The open reading frame (ORF) of a candidate *T. thermophila* Δ6 desaturase (*DES6*) was cloned from a cDNA library of strain ATCC 30007, kindly provided by Aaron Turkewitz at the University of Chicago, based on sequence information in reference 13. The nucleotide sequence of the clone is identical to THERM_00138530 found at the *Tetrahymena* genome database (<http://ciliate.org/>), except for 141 nucleotides of the predicted ORF that is missing from the cDNA clone. The actual protein sequence is 47 amino acid residues shorter than that predicted by THERM_00138530; these residues would reside between Q186 and H187 of the correct protein sequence (Fig. 1). The correction to the

original genome prediction has been confirmed by RNAseq data: the RNAseq data conform to that of our cDNA (http://tfgdgb.ihb.ac.cn/cgi-bin/gb2/gbrowse/tetrahymena/?name=THERM_00138530). Translation in *T. thermophila* recognizes only a single stop codon, TGA; the codons TAG and TAA, stop codons for most eukaryotes, are translated as glutamine in *Tetrahymena*. Ten TAG or TAA codons in the candidate desaturase were converted to CAG or CAA by overlap extension PCR (14). Overlapping portions of the sequence amplified with 11 mutagenic primers (see Table S1 in the supplemental material) were combined to serve as a template for amplification with flanking primers. After sequence verification this modified version, designated *DES6c*, was cloned into yeast expression vector pESC-HIS (Stratagene) under the control of the galactose-inducible Gal10 promoter; *DES6c* was also expressed using the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter in p423-GPD (15) and the strong, inducible Gal1 promoter (pESC-HIS). To better understand the functioning of the candidate desaturase, histidine 42, the first amino acid of a conserved HPGG motif found in Des6, was mutated to code for an alanine residue by overlap extension PCR (14), and expressed under the control of the Gal10 promoter using pESC-HIS. Predicted protein sequences for comparison were obtained from public databases (National Center for Biotechnology Information) and alignments created with Geneious software version 5.1 (Biomatters, Auckland, New Zealand).

Cloning cytochrome *b*₅s. The amino acid sequence of the *S. cerevisiae* cytochrome *b*₅ protein (ScCyb5; YNL111C) was used in a BLAST search

(16) against the *T. thermophila* genome database to identify similar cytochrome b_5 sequences. Three candidate cytochrome b_5 s, *CB5A* (TTHERM_00420210), *CB5B* (TTHERM_00051880), and *CB5C* (TTHERM_00624890) were chosen for further analysis. Codon-corrected versions, *CB5Ac* and *CB5Bc*, were synthesized for expression in *S. cerevisiae* by GENEART; the sequence representing *CB5C* was amplified from *Tetrahymena* cDNA, and its codons were corrected to standard codon usage by overlap-extension PCR (14) to yield *CB5Cc*. For comparison, the ORF of *ScCYB5* was amplified from *S. cerevisiae* cDNA, and the ORF of *AtCB5B* (At5g48810) was likewise amplified using *Arabidopsis* cDNA as a template. *Cb5A* contains duplicate cytochrome b_5 sequence motifs. Mutants of conserved residues H41A, H136A, and H41A/H136A were generated using overlap extension PCR (14) to ascertain their functions. All cytochrome b_5 cDNAs were expressed in yeast using pYES-DEST52.

Yeast expression. Strain INVSc1 of *S. cerevisiae*, which does not exhibit endogenous $\Delta 6$ desaturase activity, was transformed with the *DES6c* candidate desaturase expression construct. Next, each of the pYES:CB5 expression constructs and the empty vector pYES-DEST52 were separately transformed into these *Des6*-expressing yeast. Cultures of *S. cerevisiae* expressing genes of interest were inoculated into selective medium, incubated overnight at 30°C, and then transferred to 25 ml of selection medium containing galactose and supplemented with 1% dimethyl sulfoxide (DMSO) and 0.2 mM 18:2^{Δ9,12} fatty acid (Nu-Chek Prep; Elysian, MN). Cultures were grown at 23°C for 24 h, after which 5 ml of yeast culture was harvested for fatty acid derivatization and analysis. Each feeding experiment was performed in triplicate. We transformed the cytochrome b_5 deletion strain AMY1a (17) with the pESC-HIS-*DES6c* expression construct.

Fatty acid extraction, derivatization, and analysis. Yeast cells were washed twice with 1% DMSO in water, and total fatty acids were derivatized to form fatty acid methyl esters (18), followed by extraction into 100 μ l of hexane. The hexane extract was analyzed via gas chromatography performed on an Agilent 6890 series gas chromatograph system equipped with an EC-WAX column (30 m by 0.53 mm by 1.2 μ m; Grace Davison Discovery Science, Deerfield, IL). For gas chromatography-mass spectrometry (GC-MS) analysis, fatty acid methyl esters were dried under nitrogen gas, derivatized using 4,4 dimethyloxazoline (19), and then resuspended in 100 μ l of hexane for GC-MS performed on a Hewlett-Packard 6890 series gas chromatograph equipped with a 30-m AT-Wax column (Alltech, Deerfield, IL) coupled to an HP 5973 mass spectrometer. MS data were compared to previously published results (20) and publicly available data (<http://lipidlibrary.aocs.org/>).

Split ubiquitin growth assays. The physical interaction between *Des6* and each of the selected cytochrome b_5 proteins was tested in yeast with the mating-based/split-ubiquitin system (21). The *DES6c* ORF was cloned in-frame with the C-terminal domain of ubiquitin+PLV transcription factor contained in pMetYC-DEST, under the control of the methionine-repressible *Met25* promoter. The ORFs of *AtCB5B*, *ScCYB5*, and *CB5Ac* were cloned as fusions with the N-terminal domain of ubiquitin-encoded pNX32-DEST. The vector expressing *Des6*-ubiquitin was transformed into strain THY.AP4, while the cytochrome b_5 fusion constructs were individually transformed into the *S. cerevisiae* strain THY.AP5 (20). As a negative control, the KAT construct (21), expected to have no interaction with *Des6*, was also transformed into THY.AP5.

Each of the yeast strains harboring a cytochrome b_5 fusion construct or the negative control construct was separately mated with THY.AP4 expressing the *Des6*-ubiquitin fusion, with appropriate selection, and the resulting populations of diploid cells were used to inoculate liquid cultures. Exponentially growing liquid cultures were sampled, and the yeast cell concentration was determined using a hemocytometer. Appropriate 10-fold dilutions representing equal cell numbers of each yeast strain were spotted onto minimal medium without supplement, selecting for the activation of genes complementing the *his3* and *ade2* auxotrophies of the yeast parent. Because *Des6* expression is under the control of the methi-

onine-repressible promoter, growth on higher concentrations of methionine indicates stronger protein-protein interactions, so serial dilutions were plated on SD minimal medium containing 0, 5, or 10 μ M methionine and photographed after 3 days of incubation. In addition, β -galactosidase assays of the three cytochrome b_5 -expressing yeast cultures, plus the KAT-negative control, were conducted essentially as described previously (21).

RESULTS

Identification and characterization of the *T. thermophila* $\Delta 6$ desaturase. The candidate $\Delta 6$ desaturase (*DES6*) that we cloned from *T. thermophila* exhibits low overall identity to the sequences of previously characterized $\Delta 6$ desaturases from *Mortierella alpina*, *Homo sapiens*, and *Borago officinalis* (see Table S2 in the supplemental material), and the sequence was notably shorter than comparable desaturases (Fig. 1). However, the predicted 352-amino-acid sequence from *T. thermophila* exhibits three histidine motifs—HxxxH, HxxHH, and QxxHH—that are highly conserved in membrane-bound desaturases (22) (Fig. 1). Analysis of *Des6* with TMHMM v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) indicated that predicted transmembrane domains found in *Des6* are consistent with those found in other $\Delta 6$ desaturases. The sequence also encodes an N-terminal cytochrome b_5 -like domain, which is characteristic of a subset of eukaryotic fatty acid desaturases, including $\Delta 6$ desaturases (Fig. 1). When the predicted protein sequence of *Des6* was used to search for homologous sequences, a group of related proteins was identified among unicellular eukaryotes. The *Des6* sequence also had homology to two enzymes already shown to be $\Delta 6$ desaturases, one in *Glossomastix* and another in *Thamnidium* (see Fig. S1 in the supplemental material).

Because *Tetrahymena*, like other ciliated protists, uses protein translation codons for amino acids that are recognized by most eukaryotes as stop codons, the nucleotide sequence of the ORF was modified using overlap-extension PCR (14) to replace TAG and TAA codons representing glutamine in *Tetrahymena* (23) with glutamine codons recognized by yeast (CAG and CAA). The activity of the candidate desaturase was ascertained by expression in *S. cerevisiae*, which has no endogenous $\Delta 6$ activity, under the control of the galactose-inducible *Gal10* promoter.

When galactose-induced yeast cultures expressing *Des6* were supplemented with substrate for the $\Delta 6$ reaction, linoleic acid (18:2^{Δ9,12}), 4% of the 18:2^{Δ9,12} taken up by the yeast was desaturated to γ -linolenic acid (18:3^{Δ6,9,12}; Fig. 2A); a control strain with the empty vector did not produce detectable desaturation. These results confirm that the *DES6* gene does encode a $\Delta 6$ desaturase. A core HPGG heme-binding motif found in *Des6* is necessary for activity of other desaturases containing a cytochrome b_5 -like domain (24). When His-42 of the HPGG motif in *Des6* was mutated to an alanine residue, desaturase activity was reduced from the 4% substrate conversion activity produced by *DES6* to no activity, equivalent to the empty vector control, demonstrating the necessity of the HPGG motif in the cytochrome b_5 -like domain. When we expressed *Des6* in the *Saccharomyces* cytochrome b_5 knockout strain AMY1a (17), desaturase activity dropped to only 1% conversion of the substrate. The low 4% of conversion of linoleic acid to γ -linolenic acid seen in yeast suggested that *Des6* was not very effective in the heterologous expression system; even when expressed under the control of the strong inducible *Gal1* or constitutive *GPD* promoters, no increase in 18:2^{Δ9,12} desaturation was detected (data not shown).

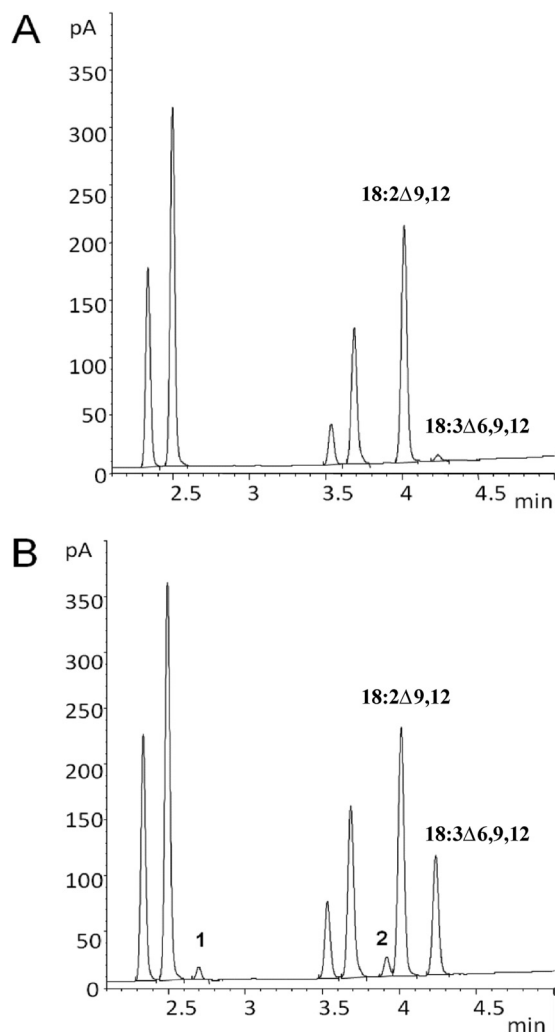


FIG 2 Gas chromatogram of fatty acid methyl esters from yeast cells supplemented with linoleic acid ($18:2^{\Delta 9,12}$). (A) Yeast cells expressing Des6 alone convert 4% of the incorporated linoleic acid to γ -linolenic acid ($18:3^{\Delta 6,9,12}$). (B) Yeast cells expressing both Cb5A and the Des6 desaturase convert 40% of linoleic to γ -linolenic acid. Novel desaturation products 1 and 2 are discussed in the text (see also Fig. 4).

Identification of cytochrome b_5 s. Fatty acid desaturases are known to receive electrons directly from cytochrome b_5 s. Three candidate cytochrome b_5 s were identified from the *T. thermophila* genome database by their amino acid sequence similarity to ScCyb5, using a BLASTP search (16). Two of the candidate genes, *CB5A* and *CB5B* (THERM_00420210 and THERM_00051880, respectively), code for proteins containing the core HPGG heme-binding motif, while *CB5C* (THERM_00624890) encodes a variant HPPG sequence; all three candidates include other amino acids strongly conserved in cytochrome b_5 proteins (Fig. 3) (25). Each of the predicted proteins has some identity with ScCyb5: 41% identity for *CB5A*, 42% identity for *CB5B*, and 38% identity for *CB5C*. The predicted protein sequence of *CB5A* is unusual because it is 214 amino acids, approximately twice the size of most cytochrome b_5 proteins, and contains two cytochrome b_5 motifs (Fig. 3). When the predicted protein sequence of *CB5A* was used to search for homologous sequences, a group of proteins with

similar duplicated cytochrome b_5 domains were identified among unicellular eukaryotes (see Fig. S1 in the supplemental material); no other cytochrome b_5 proteins with duplicate domains were detected.

Coexpression of cytochrome b_5 s with Des6. When we overexpressed the *Saccharomyces* cytochrome b_5 in the Des6-expressing yeast, we observed an increase in conversion to 8.2% (Table 1). When the *Tetrahymena* Cb5C was coexpressed in the Des6-expressing yeast line, there was no effect on $\Delta 6$ desaturation (Table 1). However, the results were dramatically different for coexpression of the other two cytochrome b_5 s: when Cb5B was coexpressed, 25% of the incorporated linoleic acid was desaturated to γ -linolenic, and the conversion was even higher with Cb5A expression, when 40% of the substrate was converted to γ -linolenic acid (Fig. 2B and Table 1). Cb5A atypically contains two cytochrome b_5 domains (Fig. 3). When proteins specifically mutagenized in the conserved cytochrome b_5 motifs (H41A and H136A) were coexpressed with Des6, desaturase activity was unaffected by the H41A mutation, but the increased desaturase activity seen when Cb5A and Des6 are coexpressed was abolished by the H136A mutation (see Table S3 in the supplemental material), indicating that it is this second cytochrome b_5 domain that provides electrons to the desaturase.

GC-MS analysis of desaturation products. Although routine analysis of fatty acid methyl esters by GC is reliable, there are variants of $\Delta 6$ -like proteins with alternate activities (26). In addition, when a *Tetrahymena* cytochrome b_5 was coexpressed with Des6, high desaturase activity produced two uncharacterized minor peaks (Fig. 2B). To establish the identity of the primary desaturation product and to identify the minor peaks, the fatty acid desaturation products were analyzed by GC-MS. Analysis of the 4,4-dimethyloxazoline (DMOX) derivative of the primary desaturation product produced a mass spectrum that corresponded to that found in the AOCS lipid library (<http://lipidlibrary.aocs.org/>) for γ -linolenic acid. The high abundance of m/z 113 and 126 are characteristic of fatty acid DMOX derivatives (20), and the observed molecular ion of m/z 331 is as expected (Fig. 4A). The 12-atomic-mass-unit (amu) difference between m/z 194 and 206 and m/z 234 and 246 indicate double bonds at the 9- and 12-carbon positions, respectively, and the peaks m/z 167 and 180 are characteristic of a $\Delta 6$ double bond (Fig. 4A) (20). These data confirm the primary desaturation product is γ -linolenic acid ($18:3^{\Delta 6,9,12}$).

The DMOX derivatives of each of the two minor peaks (Fig. 4B) exhibited a 12-amu difference between m/z 194 and 206, indicating a double bond at the ninth carbon, with an ion pair of m/z 167 and 180 consistent with a $\Delta 6$ double bond. Peak 1 (Fig. 4B) had a retention time of 2.7 min, slightly longer than $16:1^{\Delta 9}$; its DMOX derivative exhibited a molecular ion at m/z 305, and its fragmentation pattern confirmed that it was $16:2^{\Delta 6,9}$ (Fig. 4B). Peak 2 had a retention time of 3.9 min, slightly longer than $18:1^{\Delta 9}$; its DMOX derivative had a molecular ion of m/z 333, indicating an 18-carbon fatty acid containing two double bonds, and its fragmentation pattern confirmed that it was $18:2^{\Delta 6,9}$ (Fig. 4C).

Expression of a plant cytochrome b_5 with Des6. To test whether a plant cytochrome b_5 could efficiently transfer electrons to Des6, an *Arabidopsis* cytochrome b_5 , AtCb5B (At5g48810) was coexpressed with the $\Delta 6$ desaturase in yeast. Surprisingly, coexpression of AtCb5B also increased desaturase activity, resulting in almost 40% desaturation of the $18:2^{\Delta 9,12}$ substrate (Table 1).

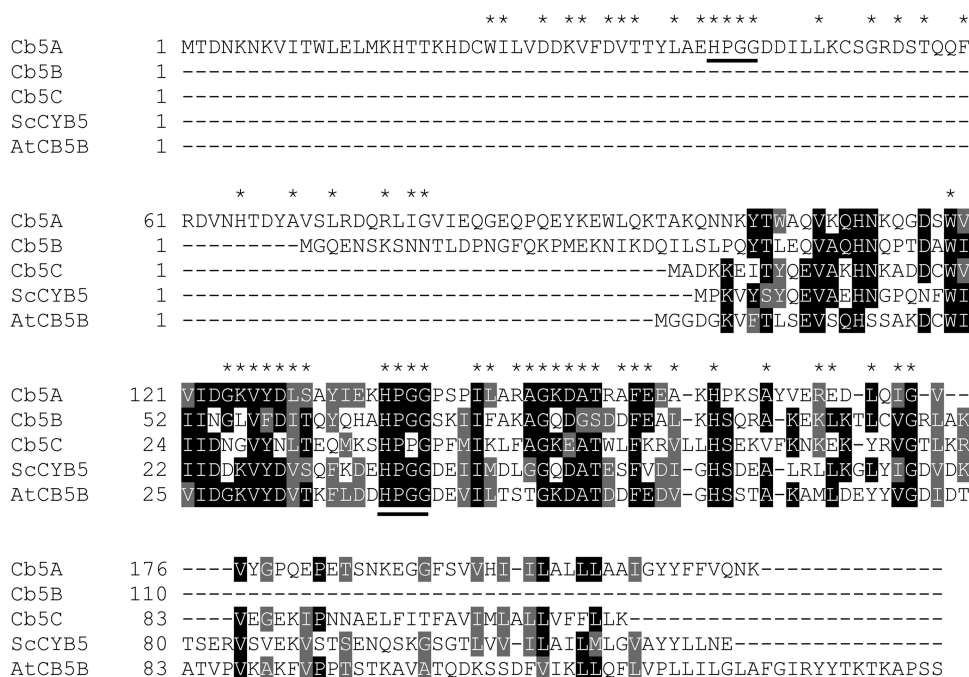


FIG 3 Sequences of the candidate cytochrome *b*₅s from *T. thermophila*. Amino acid sequence comparisons of three candidate cytochrome *b*₅s were identified from the *T. thermophila* genome database and are designated as Cb5A (THERM_00420210), Cb5B (THERM_00051880), and Cb5C (THERM_00624890), with cytochrome *b*₅ sequences of *S. cerevisiae* (ScCyb5, AAA67468) and *Arabidopsis* (AtCb5B, NP_199692). Conserved HPGG motifs are underlined. Note the second HPGG domain in Cb5A. Residues marked with an asterisk (*) are those where Cb5A matches the cytochrome *b*₅ consensus sequence (25).

Split ubiquitin growth assay. To characterize the interaction of Des6 with selected cytochrome *b*₅s, we used the split-ubiquitin growth assay for *in vivo* detection of membrane protein interactions. The ORF representing Des6 was cloned into a bait vector, fusing it to the C-terminal fragment of the ubiquitin+PLV transcription factor (21). Three cytochrome *b*₅s (AtCb5B, ScCyb5, and Cb5A) were individually cloned into the prey plasmid, fusing them to the N-terminal region of ubiquitin. When expressed fusion proteins interact, the expression of β-galactosidase is activated; the resulting enzyme activity is one measure of protein-protein interaction. A second measure of interaction is obtained because the reconstitution of ubiquitin allows release of PLV+TE, which migrates to the nucleus and triggers a cascade driving expression of prototrophic markers in the cells, so that yeast expressing bait/prey proteins with positive interactions are able to grow on minimal media (21). The split ubiquitin assay indicated that Des6 differentially interacts with each of the cytochrome *b*₅s tested (Fig. 5).

The expression of Des6 is under the control of the methionine-

repressible Met25 promoter: in the presence of increasing concentrations of methionine, expression is reduced, and lower levels of bait can distinguish between strong and weak interactions (21). Serial dilutions of the bait plus prey yeast cultures tested across a range of methionine concentrations compare the interactions with two dimensions of stringency. In the case of the Des6 desaturase, at 0 μM methionine (the first panel of Fig. 5A), the expression of the *Tetrahymena* cytochrome *b*₅ produces more abundant growth at lower dilutions, particularly relative to expression of the *Saccharomyces* cytochrome *b*₅, indicating that Des6 interacts preferentially with the *Tetrahymena* Cb5A protein. When the stringency of the interaction assay is increased by supplementation with methionine (5 and 10 μM panels, Fig. 5A), the difference is even more evident. At 10 μM methionine, growth of the strain expressing ScCyb5 is greatly reduced, even at the highest cell concentrations, while the *Tetrahymena* cytochrome *b*₅ interacts with the desaturase with sufficient strength to support strong growth. In these assays the AtCb5B protein exhibited intermediate levels of interaction. Similar levels of interaction were reflected in the β-galactosidase assays conducted using the same strains (Fig. 5B); the interaction of ScCyb5 with Des6 produces substantially less β-galactosidase activity than the Cb5A from *Tetrahymena*, only twice that of the negative control, KAT-1 (21) in the experiment.

DISCUSSION

Δ6 desaturases are broadly distributed in organisms that produce polyunsaturated fatty acids, and Δ6 enzyme activity makes a significant contribution to polyunsaturated fatty acids in the marine food chain. The activity of these desaturases has implications beyond its ecological importance, since humans obtain polyunsaturated fatty acids from their diet. Some of the richest sources of

TABLE 1 Des6 activity increases when cytochrome *b*₅ proteins are coexpressed^a

Des6 plus	% Converted ± SE
Vector	3.8 ± 0.5
ScCyb5	8.2 ± 0.9
Cb5A	42.0 ± 3.0
Cb5B	23.0 ± 2.2
Cb5C	3.8 ± 0.4
AtCb5B	39.0 ± 3.3

^a Conversion of 18:2^{Δ9,12} to 18:3^{Δ6,9,12} as a percentage of the total.

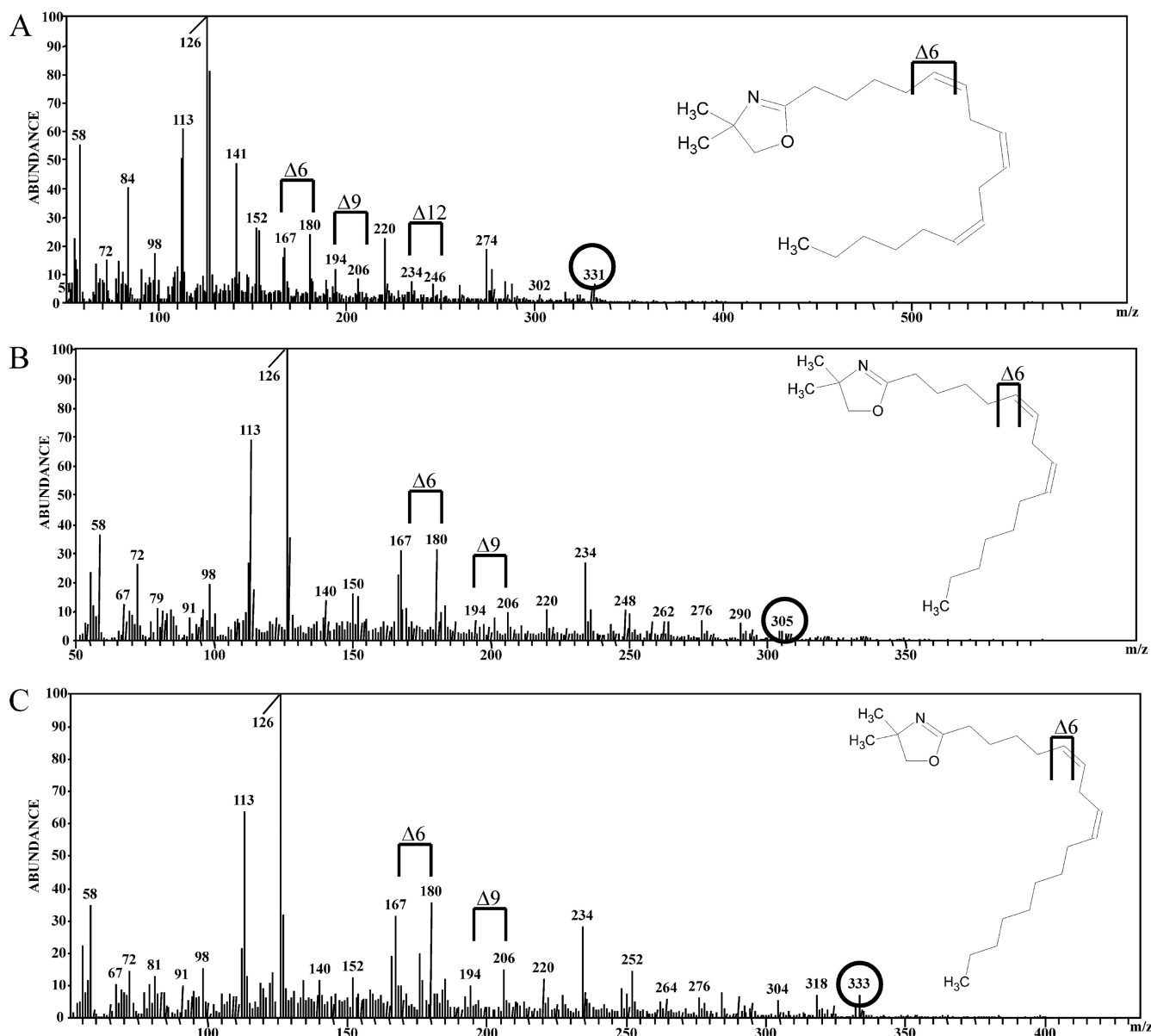


FIG 4 Verification of fatty acid structures by GC-MS. Total fatty acids from yeast cells expressing both Des6 and Cb5A were converted to 4,4-dimethyl-2-oxazoline (DMOX) derivatives. The three mass spectra shown are for fatty acids not detected in control samples. Theoretical fragmentation values and patterns are illustrated by the DMOX derivatized structures for each of the fatty acids. (A) The DMOX adduct of 18:3 $\Delta 6,9,12$ has a molecular ion of 331, a $\Delta 6$ double bond, and double bonds at $\Delta 9$ and $\Delta 12$. (B) DMOX fatty acid derivative with a molecular ion of 305, as well as $\Delta 6$ and $\Delta 9$ double bonds; this fatty acid is 16:2 $\Delta 6,9$. (C) DMOX fatty acid derivative with features similar to 16:2 $\Delta 6,9$ in panel B, but with a molecular ion of 333; this fatty acid is 18:2 $\Delta 6,9$.

these fatty acids are fish and shellfish, which in turn have acquired them by consuming marine microbes, including algae (4). Because fish populations are in decline, an important goal of metabolic engineering is to create organisms that produce fatty acids to replace this deficit (7). In order to engineer new sources of long-chain polyunsaturated fatty acids such as arachidonic acid and docosahexanoic acid, it is essential to understand not only $\Delta 6$ fatty acid desaturase activity but also how the activity of desaturases is affected by interactions between the enzymes and the electron transfer proteins of heterologous hosts.

When the ORF of the *T. thermophila* $\Delta 6$ desaturase was cloned, the predicted length was only 352 amino acids, rather than the 440

or more residues typical of the previously characterized $\Delta 6$ desaturases (Fig. 1). However, the candidate desaturase protein sequence exhibited three highly conserved histidine boxes common to membrane-bound fatty acid desaturases, as well as an N-terminal cytochrome b_5 domain characteristic of $\Delta 6$ fatty acid desaturases. The results of a search for homologous proteins in other unicellular eukaryotes identified several with similar protein sequences (see Fig. S1 in the supplemental material). It is noteworthy that the closest homologue to Des6 is a sequence from *Paramecium*. Although the activity of that predicted protein is unknown, it is small like Des6, predicted to be 343 amino acids in size. The functions of these proteins are for the most part unde-

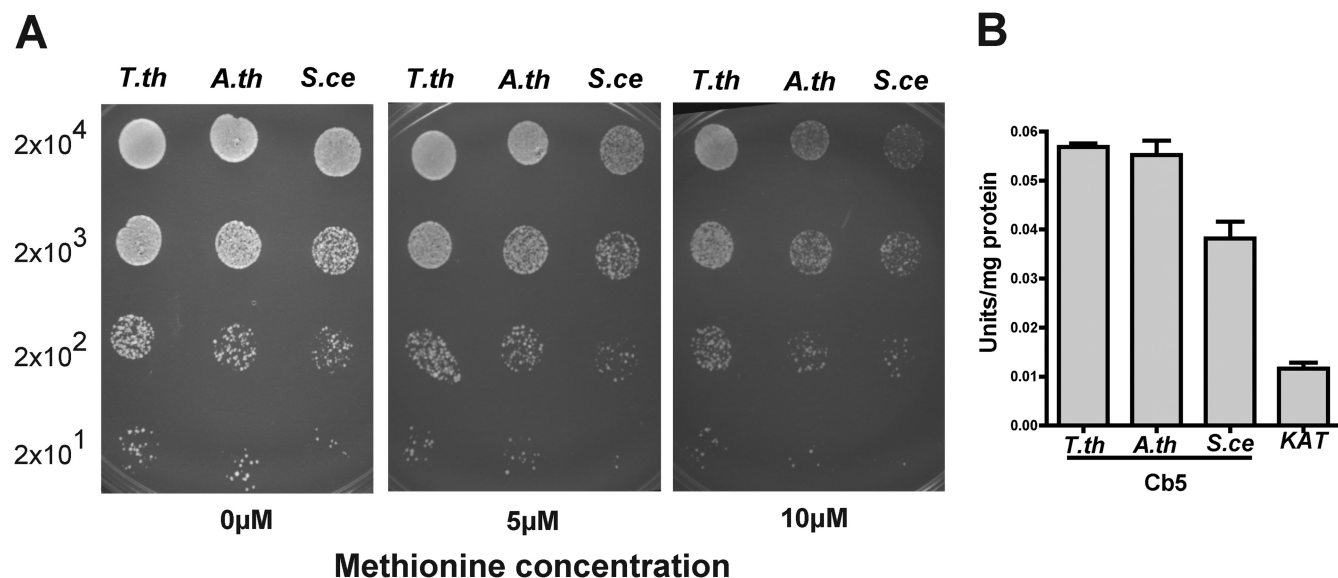


FIG 5 Interaction of Des6 with selected cytochrome b_5 s using the split-ubiquitin system. (A) Serial dilutions of diploid yeast were grown on minimal medium lacking amino acids but with increasing concentrations of methionine (0, 5, and 10 μ M) to compare the strength of interaction between Des6 and Cb5A, AtCb5B, and ScCyb5. Plates photographed after 3 days of incubation. (B) Quantitative β -galactosidase assay of yeast cells expressing Des6 with Cb5A, AtCb5B, ScCyb5, or negative control Kat-1 (21).

terminated, but the Des6 sequence does have homology to two proteins whose activity has been determined to be $\Delta 6$ desaturation, one from the marine microalga *Glossomastix* (27) and another from the filamentous fungus *Thamnidium* (28).

After the nucleotide sequence of the ORF was corrected to conform to the genetic code characteristic of yeast, the function of this $\Delta 6$ desaturase was confirmed by expression in *S. cerevisiae*. When cultures of yeast expressing the Des6 desaturase were supplemented with $18:2^{\Delta 9,12}$, a substrate for the $\Delta 6$ desaturase reaction, $18:3^{\Delta 6,9,12}$ was produced at low quantity (Fig. 2); ca. 4% of the substrate incorporated into the yeast was desaturated. Demonstration of this activity extends the range of functionally characterized $\Delta 6$ desaturases to a new group of unicellular eukaryotes.

The $\Delta 6$ desaturases are members of a larger family of membrane-bound desaturases that introduce a double bond between an existing $\Delta 9$ or $\Delta 11$ double bond and the carboxyl end of the fatty acid. The eukaryotic enzymes in this family include $\Delta 4$, $\Delta 5$, and $\Delta 8$ desaturases, which have, in addition to the conserved histidine-rich domains, an N-terminal cytochrome b_5 domain (Fig. 1) (reviewed in reference 25). Other enzymes with closely related sequences include bifunctional $\Delta 5/\Delta 6$ and $\Delta 6$ /acetylenase enzymes, as well as $\Delta 8$ sphingolipid long-chain base desaturases. The functional requirement for the cytochrome b_5 domain has been demonstrated: deletion of the domain produces loss of function, and mutations introduced at conserved residues within the domain either reduce or eliminate activity (24, 29). When we specifically mutagenized Des6 at an essential cytochrome b_5 domain residue (H42A, Fig. 1), desaturase activity was eliminated; the conserved HPGG heme-binding motif is required for desaturase activity in Des6, as it is for other desaturases containing a cytochrome b_5 domain.

Although our primary desaturase product comigrated with $18:3^{\Delta 6,9,12}$ in our GC analysis with flame ionization detection, enzymes with $\Delta 6$ -like protein sequences are known to produce $\Delta 5$

products (30), $\Delta 8$ products (31), $\Delta 8$ sphingolipid desaturation products (32) and even acetylenic triple bonds (33), rather than simple $\Delta 6$ double bonds. Using DMOX derivatization and GC-MS analysis, we confirmed the major desaturation product was in fact $18:3^{\Delta 6,9,12}$ (Fig. 4). Analysis of two minor peaks representing fatty acid derivatives at low concentrations (Fig. 4) were identified as additional $\Delta 6$ products, $16:2^{\Delta 6,9}$ and $18:2^{\Delta 6,9}$ (Fig. 4), most likely derived from $16:1^{\Delta 9}$ and $18:1^{\Delta 9}$, respectively.

In *Tetrahymena*, γ -linolenic acid can make up 19% of total fatty acids, equal to the amount of linoleic acid, suggesting that Des6 is capable of robust conversion in its parent organism (10). One explanation for the modest 4% conversion of $18:2^{\Delta 9,12}$ to $18:3^{\Delta 6,9,12}$ by Des6 expressed in yeast might be poor transcription from the promoter, but no increase in desaturation was observed when Des6 was expressed with strong inducible (GAL1) or constitutive (GPD) promoters. Indeed, when we overexpressed ScCyb5, desaturation improved only ~ 2 -fold (Table 1).

Des6 had low activity when first expressed in yeast (Fig. 2A). Two experiments showed that electron transfer by cytochrome b_5 was important. Des6 lost essentially all activity when expressed in a cytochrome b_5 knockout strain; electron transfer from the cytochrome b_5 domain of the Ole1p desaturase was not substantial, in contrast to experiments with a *Trypanosome* $\Delta 12$ desaturase (34). In addition, overexpression of the yeast cytochrome b_5 increased the desaturase activity (Table 1), indicating that electron transfer to Des6 limits the activity of the enzyme. To test whether cytochrome b_5 proteins from *T. thermophila* would more efficiently transfer electrons to Des6 and enhance its activity, three candidate cytochrome b_5 protein sequences were identified from the *Tetrahymena* genome database and cloned, and their nucleotide sequences were corrected for yeast expression. One of these candidate cytochrome b_5 s (Cb5A) was unusual in that the predicted protein was about twice the expected length and contained a doublet of apparent cytochrome b_5 domains (Fig. 3). Predicted pro-

teins that are doublet cytochrome b_5 s are very uncommon but also occur in *Paramecium*, *Leshmania*, *Oxytricha*, and *Trypanosoma* (see Fig. S2 in the supplemental material), although none of these have been functionally characterized.

Much higher desaturase activity was measured with coexpression of some of these other cytochrome b_5 proteins. Individual coexpression of *Tetrahymena* Cb5A or Cb5B proteins with Des6 dramatically increased the desaturation of 18:2 $^{\Delta 9,12}$ to 18:3 $^{\Delta 6,9,12}$. Although Des6 converted only 4% of the incorporated substrate when expressed alone in yeast, conversion increased to 25% when Cb5B was coexpressed, and conversion reached 40% when Cb5A was coexpressed (Fig. 3). These two *Tetrahymena* cytochrome b_5 s were more effective in passing electrons to the desaturase than the yeast ScCyb5, achieving in one case a 10-fold increase in activity; an *Arabidopsis* cytochrome b_5 was also very successful in augmenting Des6 activity (Table 1). Coexpression of a third *Tetrahymena* candidate cytochrome b_5 , Cb5C, had no effect on Des6 activity (Table 1). This predicted protein has HPPG instead of the canonical HPGG motif (Fig. 3), which may account for its failure to support increased Des6 activity.

Although Cb5A is the most successful in supporting Des6 desaturation, its success is not based on having two cytochrome b_5 domains to transfer electrons to the desaturase; when critical residues of each of the heme-binding domains were mutagenized (Fig. 3), only proteins with the H136A mutation lost the positive effect that Cb5A has on the activity of Des6 (see Table S3 in the supplemental material), while the H41A mutant retained activity, indicating that it is only the second cytochrome b_5 domain that supplies electrons to the desaturase. It is not known whether the other, N-terminal, domain is active in $\Delta 6$ desaturation in *Tetrahymena* metabolism or whether it has a different metabolic function in *T. thermophila*. The occurrence of predicted proteins with doubled cytochrome b_5 domains (see Fig. S2 in the supplemental material) suggests that the doublet structure may serve some function.

Since we did not quantify the amount of each Cb5 protein, some differences between the observed desaturase activities could be due to differing levels of protein produced in each strain. We used an alternate method of examining the relationship of some Cb5 proteins to the desaturase by expressing the proteins as fusions in a yeast two-hybrid system designed to characterize the physical interactions of membrane proteins, the mating-based split-ubiquitin assay (21). When we cloned and expressed the desaturase and cytochrome b_5 proteins in this second expression system, differences in the physical interactions between the various cytochrome b_5 s and the Des6 desaturase were observed that are consistent with our measurements of desaturase activity (compare Fig. 2 and Table 1 to Fig. 5). The relatively poor interaction between ScCyb5 and Des6 measured either by the sensitive assay of yeast growth or the quantitative β -galactosidase assay (Fig. 5) correlates with the low level of desaturase activity measured (Table 1). In contrast, both yeast growth and the β -galactosidase assay indicate a strong physical interaction between *Tetrahymena* Cb5A and Des6 (Fig. 5), correlated with the significant increase in desaturation (Fig. 2).

The options for electron flow are illustrated in Fig. 6. When Des6 relies on the yeast ScCb5 for electron transfer, transfer is poor and desaturase activity low (Fig. 6A). However, when the *Tetrahymena* (or *Arabidopsis*) Cb5 is coexpressed, desaturation is 10-fold higher (Fig. 6B). In this case, electrons flow from NADH via CBR either directly via the *Tetrahymena* Cb5 to Des6 or to

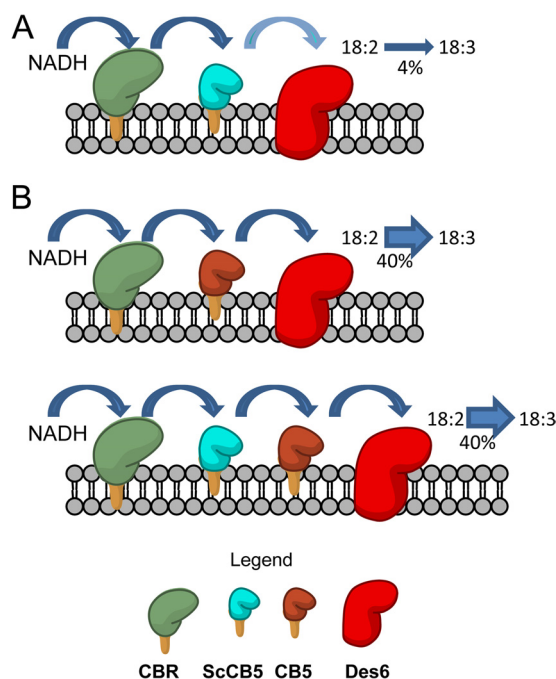


FIG 6 Models of electron transfer for $\Delta 6$ desaturation. Electrons derived from NADH are sequentially transferred to the Des6 desaturase by the yeast cytochrome b_5 reductase (CBR) via one or more cytochrome b_5 proteins. (A) When electrons from CBR are transferred inefficiently to the *Tetrahymena* Des6 via the *S. cerevisiae* cytochrome b_5 , as indicated by a light arrow, the desaturase converts only 4% of the substrate. (B) In the presence of the *Tetrahymena* cytochrome b_5 , which efficiently transfers electrons to Des6, a 10-fold increase in activity is observed. Electron transfer from the cytochrome b_5 reductase may be directly through *Tetrahymena* Cb5 to Des6, or first to the yeast ScCyb5, and then Cb5, and finally to the desaturase. Both electron transfer pathways may operate simultaneously.

ScCyb5, and then to *Tetrahymena* Cb5, and finally to the desaturase. It is possible that both pathways are at work in enhancing $\Delta 6$ fatty acid desaturation. The results of these experiments indicate that it is the interaction between cytochrome b_5 and desaturase that is important. Increased desaturase activity produced by coexpression of cytochrome b_5 proteins indicates the importance of understanding the entire desaturation electron transport chain. Although the ScCyb5 transfers electrons poorly and support little Des6 desaturation either at its natural level of expression or when overexpressed (Table 1), Des6 has much greater activity when coexpressed with two of the *Tetrahymena* cytochrome b_5 proteins, especially Cb5A (Table 1).

The interactions of cytochrome b_5 with its redox partners has been intensively examined, both with its primary electron donor cytochrome b_5 reductase (reviewed in reference 35) and with electron acceptors such as P450 enzymes (36), methemoglobin (37), and the nonphysiological model acceptor cytochrome c (38). Unfortunately, membrane-bound fatty acid desaturases have been intractable subjects for crystallization and other attempts to resolve their three-dimensional structure. It must be supposed that *Tetrahymena* Cb5 interacts with DES6 by electrostatic interactions, in a fashion similar to that of cytochrome b_5 interactions with other electron acceptors. These interactions are transient, since transfer of electrons is a rapid and evanescent process. Nevertheless, we have established the importance of the interaction

between cytochrome b_5 proteins and the Des6 desaturase. The data from these analyses support the proposal that the stronger interaction of Des6 with *Tetrahymena* Cb5A supports high rates of desaturase activity, whereas the weak interaction between Des6 and ScCyb5 is the primary cause of the low activities observed in our initial assays of the desaturase. Our results demonstrate that productive protein-protein interactions within the endoplasmic reticulum electron transport chain are critical to support desaturase activities. Understanding these interactions is particularly important to achieving high rates of desaturation from proteins expressed in heterologous hosts.

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